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The Serial Mediating Role of Acidity Content and total Soluble Solids in Linking Peel Thickness to Vitamin C Content in Some Accessions of *Citrus limon* (L.) Burm

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This study aimed to examine whether the association between PT and VC content is influenced by the levels of TSS and AT content. More Specifically, the experimental analytical investigation involved 24 lemon Accession (*Citrus limon* (L.) Burm.), with a focus on the VC content in the fruits. Serial mediation analyses were conducted using Model 6 of Hayes' PROCESS macro (version 4.2), with controls for weight, FL, number of seeds per fruits, and percentage of juice. The mediation analysis indicated that PT had a total effect of 31.74 (95% CI, 9.04-54.45) on VC content. This effect was divided into a direct effect of 7.13(95% CI, -14.15-28.40) and an indirect effect of 24.61 (95% CI, 12.16-37.93), with an estimated proportion of 77.54% being mediated by levels of TSS and AT. Additionally, TSS and AT were identified as independent factors contributing to the increase in VC content, serving as significant mediators in the relationship between PT and VC content. In summary, our findings suggest that levels of TSS and AT play crucial roles as mediators in linking PT to VC content. These findings have practical implications for optimizing vitamin C levels in citrus fruits.
Keywords: Citrus limon, mediation, path, peel thickness, vitamin C, Accessions.

INTRODUCTION

Citrus fruits from plants in the Rutaceae family (Velasco and Licciardello, 2014) are widespread ancestral crops in tropical and subtropical regions around the world (Hvarleva *et al.*, 2008). The diploid citrus fruits are widely known for their natural tendency to hybridize through cross-pollination, thus creating hybrids and increasing ploidy levels (Barrett and Rhodes, 1976; Nicolosi *et al.*, 2000). They are often recognized for their high vitamin C content, and this has prompted extensive research into the amount of vitamin C present in their pulp and juice. Genetic factors appear to play a key role in causing higher levels of ascorbic acid in oranges and lemons, followed by grapefruits and tangerines, despite the possible influence of environmental factors and agricultural practices (Dhuique-Mayer *et al.*, 2005; Cano *et al.*, 2008; Martí *et al.*, 2009; Bermejo and Cano, 2012; Bermejo *et al.*, 2016; Magwaza *et al.*, 2017) Moreover, the desire for high-quality fruits has stimulated research into breeding fruit varieties with superior characteristics Jenks and Bebeli, 2011). Citrus fruits occupy a significant place in a healthy diet due to their antioxidant properties and nutritional value. Indeed, ascorbic acid is a water-soluble vitamin that offers many benefits for human health, such as preventing scurvy, promoting collagen synthesis, healing wounds, and even improving the absorption of non-heme iron (Teucher *et al.*, 2004; Najwa and Azrina, 2017). As an antioxidant, vitamin C protects cells by neutralizing free radicals, and it is also used as a food additive (Whitney and Rolfes, 2008).
Epidemiological studies have established a direct link between the consumption of citrus fruits and a reduced risk of chronic diseases like cancer, cardiovascular diseases, and diabetes due to the therapeutic components they contain (Murthy *et al.*, 2021; Wang *et al.*, 2021). Citrus fruits have therefore gained recognition for their beneficial pharmacological effects, reinforcing their importance in the field of human health. Moreover, the essential oils extracted from citrus peels have powerful free radical scavenging and

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antifungal properties (Grosso *et al.*, 2013; Sharma *et al.*, 2019). This rich range of beneficial compounds results from the varying chemical compositions of citrus peels under the influence of various climatic conditions. The organic acids in citrus fruits also play a key role in digesting nutrients and stimulating blood circulation, and these are also key quality indicators for these fruits, because acidity is crucial to the flavor of fleshy fruits. Indeed, citrus fruits are characterized by their high level of acidity, mainly due to citric acid, with the amount varying depending on the species. For example, lemons and limes, which are classified as sour, contain high levels of citric acid (up to 60%), while sweeter varieties like oranges have much lower levels (5%). The acidity influences the genetic division of citrus fruits into acid types (e.g., lemons, limes) and intermediate types (e.g., oranges, mandarins, pomelos), and it plays a major role in the aromatic composition of fruits (Ulrich, 1970; Vandercook, 1977; Sinclair, 1984; Scheible *et al.*, 2000; Ziena, 2000; Ollitrault *et al.*, 2003; Karadeniz, 2004; Albertini *et al.*, 2006). Although organic acids are also present in the peel, they are less concentrated there than in the pulp (Ladaniya, 2008).

The lemon (*Citrus limon L.*) plays a crucial role in the Moroccan economy. Indeed, Morocco's production of limes (green lemons) and lemons (yellow lemons) has seen a 9.3% increase in yearly production since 2014. With this being 41,307 metric tons in 2019 (FAO, 2019), the country was ranked as the 32nd largest producer of limes and lemons globally according to data from the Food and Agriculture Organization of the United Nations (FAO, 2019). Lemons are naturally heterozygous, resulting in broad variability within the seedling population. Understanding the genetic diversity and phylogeny of these varieties could therefore enhance the efficiency of germplasm characterization and its application in breeding programs (Gulsen and Roose, 2001).

Moreover, lemons are very rich in essential natural compounds, including citric acid, ascorbic acid, minerals, flavonoids, and essential oils (Del Río *et al.*, 2004). Due to the significant presence of bioactive molecules, such as natural antioxidants, phenolic acids, and flavonoids, the peel that comprises approximately half of the fruit's mass has become the subject of extensive research (Dhawan, 2014). Ripening citrus fruits leads to an accumulation of sugars and pigments and reduces acidity in the flesh, so the sugar–acid ratio changes with maturity and influences the taste of the fruit. Indeed, prolonged ripening decreases acidity while intensifying sugars, thus altering the taste and durability of the fruit (El-Otmani and Zacarias, 2014). During the ripening process, changes have been observed, such as reductions in peel thickness and acidity (Sinclair, 1972; Ozeker, 2000). Additionally, the vitamin C content is influenced by skin thickness (Kaur *et al.*, 2022), while the TSS and TA parameters are also closely related (Pingle, 2011; Tilekar, 2011). Several noteworthy previous works have explored the relationships between various parameters—such as TSS, TA, VC, and so on— but to the best of our knowledge, no study has yet addressed the relationship between these parameters through a mediation analysis, as was the case with the study of Causse *et al.* (2003). Indeed, some studies (Alós *et al.*, 2021; Sayed *et al.*, 2006; Zandkarimi *et al.*, 2011; Rekha *et al.*, 2012; El-Khayat, 2019) have examined various aspects, but none of these applied a mediation approach to comprehensively understand these relationships. It will therefore be very interesting to examine lemon citrus hybrids in this study in terms of their variability and investigate how the vitamin C content could be influenced by certain physicochemical parameters.

In this context, our research hypotheses rely on using a theoretical model to examine: (i) the effect of peel thickness on total soluble solid (TSS) content, levels of acidity (TA), and vitamin C content; (ii) the mediating role of total soluble solid (TSS) and acidity content (TA) in the relationship between peel thickness and vitamin C content; and (iii) whether total soluble solid (TSS) and acidity (TA) content act as serial mediators in the association between peel thickness and vitamin content.

MATERIALS AND METHODS

***Experimental Site*:** The experiment was carried out at the El Menzeh collection of the National Institute of Agronomic Research (INRA) in Kenitra, Morocco. This site is geographically located at an altitude of approximately 25 m and at a latitude of 34°64. The climate is Mediterranean, sub-humid, and temperate in nature, with a mild winter and few frosts. The soil of the experimental site is sandy (98%) on the surface and sandy clay at depth.

***Plant materials*:** Twenty-four different lemon genotypes [*Citrus limon (L.)* Burm. F] that had been grafted onto bitter orange rootstocks (*Citrus. Aurantium L.)* were used as experimental material. The fruits were harvested during the 2019–2020 season from adult trees that were of the same age (14 years) and had received the same cultural practices.

Table 1. List of characteristics in this study.

Parameter	Abbre-viation	Range of variabiliy		Unit
		Minimum	Maximum	
Fruit weight	FW	89.65	227.39	g
Fruit length	FL	64.32	88.53	mm
Peel thickness	PT	3.30	8.35	mm
Seed number	SDN	0.00	12.00	Seeds/fruit
Vitamin C	VC	424.41	750.12	mg/L
Juice percentage	JP	26.30	43.90	%
Titratable acidity	TA	3.00	6.90	%
Total soluble solids	TSS	6.00	9.20	% °Brix

***Physical and chemical determinations*:** Data about the various physicochemical parameters of the fruits and fruit juice were collected for fruit weight (FW), fruit length (FL),

titratable acidity (TA), total soluble solids (TSS), the thickness of the fruit's peel (Predictor) (PT), the number of seeds per fruit (SDN), and the juice percentage (JP) using the proposed indicators (Table 1). The physicochemical study of the fruit samples was carried out in accordance with relevant scientific standards and methodologies. Nine fruits for each genotype were randomly collected from different directions to record different observations, and the observations were recorded for each fruit separately. Fruit weight was measured with an electric scale. Parameters like fruit length and peel thickness were measured using a digital caliper (Mitutoyo Inc., Japan). The number of seeds for each fruit were also counted. The level of total soluble solids (Potential Meditator) in the juices was analyzed using a PR-201a digital refractometer (Atago, Tokyo, Japan). Titratable acidity (Potential Meditator), meanwhile, was determined through titration with a 0.1 N NaOH solution and phenolphthalein as an indicator, with the results being expressed in grams of citric acid per 100 ml of juice. The juices were all pressed on the same day, filtered through a 1 mm mesh sieve, and stored in amber bottles at -18°C until further analysis. Each measurement comprised three repetitions. To measure the thickness of the fruits' peels, the following method was used: The fruits were cut in half horizontally at their equator before the maximum width of the entire fruit and the inner part of the fruit were measured using a caliper. The difference between these two measurements was then halved to give the thickness of the peel in millimeters (mm).

***The determination of ascorbic acid (Vitamin C) content as a prediction*:** The technique described by Izuagie A and Izuagie F (2007) was used to determine the vitamin C concentration. We dissolved 0.02 g of KIO3 and 1.06 g of KI In a 500-mL bottle of distilled water. This was then acidified by adding 1 mL of concentrated tetraoxosulphate (VI) (H3SO4) acid to the solution. After swirling the combination, the solution was topped up to 500 ml with purified water. To ensure that the contents were homogeneous, each bottle was plugged and agitated. Consequently, the iodine concentration was 5.6076 x 10-3 mol. L-1. Each sample was titrated with 20 mL of juice against this standard iodine solution. To get an indication, a starch solution was used. Each measurement comprised three replications, each of which used juice from three different fruits, so a total of nine fruits were used per measurement.

***Covariates*:** Confounding factors linked to the thickness of the peel (PT), the total soluble solids (TSS) content, the acidity content (TA), and the vitamin C content (VC) were considered as covariates in this study. Based on a study and review of the literature (Zandkarimi *et al.*, 2011; Usman *et al.*, 2012, 2020), we selected the following confounding factors: average fruit weight, percentage in juice, fruit length, and the number of seeds per fruit.

***Statistical analysis*:** All the study data were first analyzed using the SPSS version 25 software (SPSS, 2017) in order to carry out a descriptive analysis to show the distribution of the studied physicochemical characteristics. The parametric ANOVA test and multiple comparisons of means through Bonferroni's test were applied to estimate the significance of the differences between the examined parameters. All the p-values presented were two-sided, while $p < 0.05$ was considered statistically significant. To explore whether TSS and TA content influence the association between peel thickness and vitamin C content, mediation analyses were performed. Mediation occurs when an independent variable (X) influences a dependent variable (Y) through one or more mediators (M) (Preacher and Hayes, 2008). The statistical analysis followed the recommendations of Hayes'(Preacher and Hayes, 2008). Figure 1 illustrates the mediation model for Model 6 of the PROCESS macro (version 4.2) of Hayes (Hayes, 2012). The models were examined using 5000 bootstrap samples to allow repeated bootstrapping in all analyses, as recommended by Hayes (Hayes, 2018). For more details, refer to the work of Lemardelet and Caron (2022).

To determine the significance of the indirect effect, the bootstrap method was used. This method uses random sampling to estimate the sampling distribution of almost any statistic, thus allowing the parameters of interest to be calculated. Obtaining these parameters in turn makes it possible to obtain their product and calculate the indirect effect of mediation. Calculating this indirect effect through bootstrapping also allows the estimation of confidence intervals and standard errors for the desired effect (Efron and Tibshirani, 1994). This method is recommended over other methods because it follows the empirical distribution of the indirect effect (non-normal), resulting in greater statistical power(Caron, 2019; Özdil and Kutlu, 2019), a more appropriate type I error rate (Caron, 2019), and robustness with data that is not normal (Cheung and Lau, 2008). In this context, our study adopted a serial mediation model (Fig. 1) that includes two mediators, namely the total soluble solids and acidity content. The serial mediation model encompasses multiple paths, with each representing a regression relationship (Fig. 1).

Path a represents the regression of PT on TSS. Path b represents the regression of PT on AT. Path c represents the regression of TSS on AT while controlling for the effect of PT. Path a' represents the regression of TSS on VC content while controlling for the effect of PT. Path b' represents the regression of acidity on VC content while controlling for the effects of PT and TSS content. Path D represents the total effect, or in other words, the regression of PT on vVC content. Path d represents the direct effect, thus reflecting the influence of PT on VC content while controlling for the effects of TSS and AT. Estimating these parameters required three regression analyses, with each corresponding to a specific step in the serial mediation analysis. The initial step involved regressing PT on TSS to obtain parameter a. The subsequent steps comprised regressing PT and TSS on AT to obtain parameters b and c, respectively. Finally, PT, TSS, and

AT were regressed on VC content to get parameters d, a', and b'. An optional fourth step involved regressing PT on VC content to obtain D, the total effect, which can also be calculated by summing up all the primary indirect effects (aa', bb', acb') and the direct effect (D = d + aa' + bb' + acb'). When considering the two mediators, the total effect D is divided into five distinct indirect effects, three of which are primary indirect effects.

- The specific indirect effect of TSS, denoted by the product aa', as depicted in Fig. 1.
- The specific indirect effect of AT, indicated by the product bb', as represented in Fig. 1.
- The indirect series effect of TSS and AT, encapsulated by the product acb', as shown in Fig.1.
- Additionally, two secondary indirect effects are present:
- The specific indirect effect of TSS, characterized by the product ac, as depicted in Fig.1.
- The specific indirect effect of AT, represented by the product cb', as illustrated in Fig. 1.

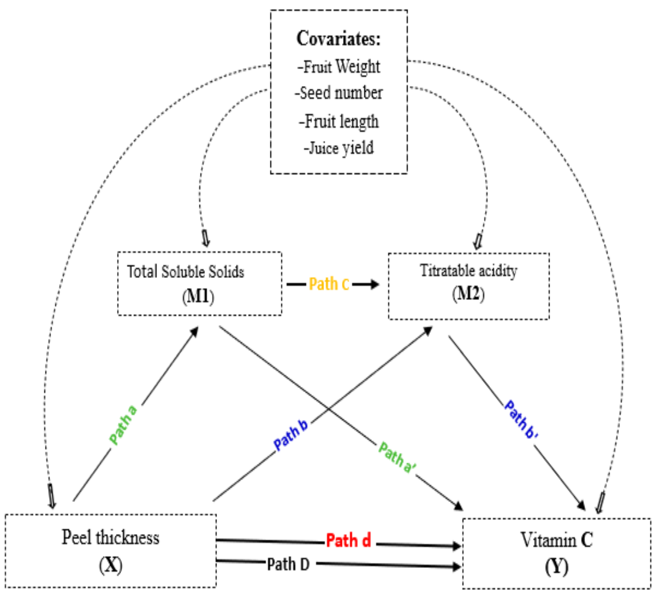

Figure 1. An illustration of the total effect (Path D), indirect effects (Path a, a′, b, b′ and c), and direct effect (Path d) on the relationship between the exposure (X) with outcome (Y) via the mediators (M1 and M2).

The three primary indirect effects are collectively categorized as the total indirect effect. If this effect significantly deviates from zero, it indicates the presence of at least one mediation effect in the model. Thus, it is essential to assess whether the serial indirect effect, **acb'**, significantly differs from zero, thereby indicating a serial mediation effect. Indeed, if this is not statistically significant, further examination of other indirect effects becomes necessary. The absence of a significant relationship between TSS and AT content may suggest parallel mediation or perhaps a straightforward indirect effect stemming from a single mediator.

RESULTS

Analyzing the Variability of the Characteristics Measured for the Lemon Genotypes:

Phenotypic characterization of the fruit (Table 2): The results of the analysis of variance revealed significant differences among the eight studied parameters, with the significance level being set at $p<0.05$. Tukey's multiple mean analysis identified significant differences between genotypes for each of the studied physicochemical parameters. Upon analysing the different parameters, several noteworthy observations become apparent. Concerning FW, the *Remontant* (199.32±24.34g), *Villafranca* (197.23±9.45g), and *Vernia* (193.07±7.54g) genotypes stand out with significantly higher weights compared to other varieties, with them showing a total average of 143.38±29.13g, a minimum value of 89.65g, and a maximum value of 227.39g.

Regarding FL, the *Villafranca* (83.34±4.32mm), *Remontant* (79.22±8.53mm), and *Maghzalani* (75.77±5.54mm) genotypes exhibit significantly higher values, with a total average of 75.45±6.27mm, a minimum value of 64.32mm, and a maximum value of 88.53mm. For PT, the *Kerkachi* (8.23±0.15mm), *Vernia* (7.18±0.45mm), and *Portugal* (7.20±0.81mm) genotypes distinguish themselves with significantly higher values compared to the other varieties, with them having a total average value of 5.84±1.10mm, a minimum value of 3.30mm, and a maximum value of 8.35mm. Concerning seeds number per fruit, the *Vernia* (11.92±0.14), *Remontant* (8.25±0.46), and *Eureka* (8.58±0.22) varieties present significantly higher values, with a total average of 4.92±2.63, a minimum value of 0.00, and a maximum value of 12.00 seeds per fruit. For juice percentage, the *Kerkachi* (31.6±0.5%), *Genes* (36.41±0.3%), and *Portugal* (28.98±0.1%) genotypes exhibit notably lower values compared to other varieties, with a total average of 34.66±4.35%, a minimum value of 26.3%, and a maximum value of 43.9%. Analysing the VC content across different genotypes reveals some notable variations. Varieties such as *Ba Ahmed* (733.67±5.7a), *Vernia* (694.19±5.7b), *Genes* (648.13±5.7cd), *Kerkachi* (743.54±5.7a), *Lisbonne* (687.61±5.7b), *Lunari* (608.65±5.7e), *Maghzalani* (641.55±9.87d), *Malti* (641.55±0d), *Portugal* (690.9±0b), *PSP* (736.96±5.7a), *Saasly* (654.71±5.7cd), and *Sicile* (664.58±5.7c) stand out with significantly higher values, thus indicating elevated VC levels. On the other hand, *Dellys* (493.5±0g), *Valence* (503.37±0g), and *Villafranca* (427.7±5.7h) exhibit lower values, so these genotypes have comparatively less VC content. The remaining varieties fall within these extremes.

The vitamin C content varies considerably, with the total average being 610.02±83.66 mg/L, the minimum value being 424.41 mg/L, and the maximum value being 750.12 mg/L. Regarding TSS, the *Ba Ahmed* (8.60±0.60% °Brix), *Vernia* (8.50±0.18% °Brix), and *Kerkachi* (7.90±0.80% °Brix) varieties stand out with significantly higher values, while *Valence* (6.40±0.05% °Brix) has a notably lower value. The total average for all accessions is 7.26±0.65% °Brix, with a minimum value of 6.00% °Brix and a maximum value of 9.20% °Brix.

Notably, *Vernia* (6.8±0.1%) exhibited the highest AT, followed closely by *Ba Ahmed* (6.15±0.15%) and *Kerkachi* (6.10±0.10%). On the lower end of the AT scale were the *Lunari* (3.10±0.10%), *Eureka* (3.2±0.17%), and *Corregia* (3.05±0.05%) genotypes. The average AT across all Accessions was 4.95±1.18, with a minimum value of 3.00% and a maximum value of 6.90% (Table 1 & 2).

Note: Path a (mediating model of TSS): PT's relationship with TSS; Path a' (model result for TSS): the relationship between TSS and vitamin C content; Path b (mediating model for high levels of TA): the relationship between PT and high levels of AT; path b' (outcome model for high BP levels): the relationship between high BP levels and vitamin C content; Path c (mediating model): the relationship of TSS with high levels of TA; Path D: the relationship between the thickness of the fruit peel and vitamin C content. Abbreviations: TSS, Total soluble solids; TA, titratable acidity; 95% CI, 95% confidence interval; A — Adjusted for average fruit weight, fruit length, number of seeds per fruit, juice percentage, acidity content; B — Adjusted for average fruit weight, fruit length, number of seeds per fruit, juice percentage, acidity content (TA); C — Adjusted based on average fruit weight, fruit length, number of seeds per fruit, juice percentage, total soluble solids (TSS), and titratable acidity (TA); D — Adjusted according to the average weight of the fruit, the length of the fruit, the number of seeds per fruit, the juice percentage, the thickness of the peel, the acidity content; E — Adjusted for peel thickness, average fruit weight, fruit length, number of seeds per fruit, juice percentage and total soluble solids (TSS); F — Adjusted for average fruit weight, fruit length, number of seeds /fruit, juice percentage, total soluble solids (TSS), titratable acidity and peel thickness. *** p < 0.001. Abbreviations: 95% CI = 95% confidence interval. If the CI contains zero, the indirect effect is not significant.

Table 2. Continued…

Varieties	FW (g)	FL (mm)	PT (mm)	SDN
Lunari	109.7±20.75fgh	79.12±7.45abcde	3.55±0.23 j	0.92±0.14k
Panaché	115.19±19.17 efgh	67.11±2.84ef	5.61±0.20cdefg	5.33±0.88efg
Eureka	116.15±10.71efgh	71.72±5.21bcde	5.72±0.55cdefg	8.58±0.22b
Kennedy	119.5é±14.44efgh	68.63±5.45e	4.5±0.47ghij	4.19±0.17fghi
Lisbonne	120.36±12.25defgh	71.72±2.69bcde	4.92±0.30fghi	3.11±0.19hij
Quatre Saisons	123.89±12.16cdefgh	69.52±3.94de	5.32±0.23efgh	2.44±0.51 j
Genes	125.26±14.49cdefgh	76.03±3.9abcde	5.27±0.15efgh	2.69±0.34j
Lisbonne epineux	126.46±3.02cdefgh	70.95±2.21cde	6.04±0.50bcdef	4.11±0.19ghi
Kerkachi	126.74±1.56cdefgh	75.42±2.29abcde	8.23±0.15a	5.30±0.05efg
Doux acide	127.10±15.02cdefgh	67.83±3.72e	5.30±0.46efgh	2.92±0.36ij
Portugal	128.69±6.92cdefgh	74.28±2.19abcde	7.20±0.81ab	5.44±0.51def
Corregia	129.11±12.11cdefgh	73.87±4.53bcde	4.18±0.34hij	0.10±0.17k
Maghzalani	132.4±27.76cdefgh	75.77±5.54abcde	6.87±0.58bc	8.25±0.46b
Malti	144.62±13.32cdefg	73.79±5.15bcde	5.74±0.56cdefg	6.31±0.34cde
Arbi	144.87±14.31cdefg	71.4±2.64bcde	4.9±0.29fghi	2.56±0.51j
Dellys	152.06±19.15bcdef	75.34±1.92abcde	5.42±0.20defg	3.0±0.30ij
Sicile	152.6±13.45abcdef	78.55±5.10abcde	5.88±0.13bcdef	6.78±0.19c
Saasly	158.66±21.09abcde	77.41±2.4abcde	6.1±0.09bcdef	2.75±0.43j
BA AHMED	161.68±3.44abcde	76.45±4.08abcde	6.74±0.33bcd	6.11±0.19cde
Valence	167.36±25.11abcd	83.34±4.32abc	6.48±0.65bcde	4.31±0.34fgh
PSP	168.98±8.97abc	84.12±2.25ab	6.01±0.51bcdef	6.25±0.8cde
Vernia	193.07±7.54ab	87.23±1.25a	7.18±0.45ab	11.92±0.14a
Villafranca	197.23±9.45ab	81.96±3.61abcd	6.54±0.52bcde	4.53±0.24fg
Remontant	199.32±24.34a	79.22±8.53abcde	6.58±0.76bcde	8.25±0.46b
Moyen	143.38±29.13	75.45±6.27	5.84±1.10	4.92±2.63

Note: Where FW = Fruit weight, FL = Fruit length, PT = Peel thickness, SDN = Number of seeds. Averages that do not share the same letter are significantly different at the 0.05 level. Data are averaged ± SD.

Table 2. Continued…

Varieties	FW (g)	FL (mm)	PT (mm)	SDN
Lunari	109.7±20.75fgh	79.12±7.45abcde	3.55±0.23 j	0.92±0.14k
Panaché	115.19±19.17 efgh	67.11±2.84ef	5.61±0.20cdefg	5.33±0.88efg
Eureka	116.15±10.71efgh	71.72±5.21bcde	5.72±0.55cdefg	8.58±0.22b
Kennedy	119.5é±14.44efgh	68.63±5.45e	4.5±0.47ghij	4.19±0.17fghi
Lisbonne	120.36±12.25defgh	71.72±2.69bcde	4.92±0.30fghi	3.11±0.19hij
Quatre Saisons	123.89±12.16cdefgh	69.52±3.94de	5.32±0.23efgh	2.44±0.51 j
Genes	125.26±14.49cdefgh	76.03±3.9abcde	5.27±0.15efgh	2.69±0.34j
Lisbonne epineux	126.46±3.02cdefgh	70.95±2.21cde	6.04±0.50bcdef	4.11±0.19ghi
Kerkachi	126.74±1.56cdefgh	75.42±2.29abcde	8.23±0.15a	5.30±0.05efg
Doux acide	127.10±15.02cdefgh	67.83±3.72e	5.30±0.46efgh	2.92±0.36ij
Portugal	128.69±6.92cdefgh	74.28±2.19abcde	7.20±0.81ab	5.44±0.51def
Corregia	129.11±12.11cdefgh	73.87±4.53bcde	4.18±0.34hij	0.10±0.17k
Maghzalani	132.4±27.76cdefgh	75.77±5.54abcde	6.87±0.58bc	8.25±0.46b
Malti	144.62±13.32cdefg	73.79±5.15bcde	5.74±0.56cdefg	6.31±0.34cde
Arbi	144.87±14.31cdefg	71.4±2.64bcde	4.9±0.29fghi	2.56±0.51j
Dellys	152.06±19.15bcdef	75.34±1.92abcde	5.42±0.20defg	3.0±0.30ij
Sicile	152.6±13.45abcdef	78.55±5.10abcde	5.88±0.13bcdef	6.78±0.19c
Saasly	158.66±21.09abcde	77.41±2.4abcde	6.1±0.09bcdef	2.75±0.43j
BA AHMED	161.68±3.44abcde	76.45±4.08abcde	6.74±0.33bcd	6.11±0.19cde
Valence	167.36±25.11abcd	83.34±4.32abc	6.48±0.65bcde	4.31±0.34fgh
PSP	168.98±8.97abc	84.12±2.25ab	6.01±0.51bcdef	6.25±0.8cde
Vernia	193.07±7.54ab	87.23±1.25a	7.18±0.45ab	11.92±0.14a
Villafranca	197.23±9.45ab	81.96±3.61abcd	6.54±0.52bcde	4.53±0.24fg
Remontant	199.32±24.34a	79.22±8.53abcde	6.58±0.76bcde	8.25±0.46b
Moyen	143.38±29.13	75.45±6.27	5.84±1.10	4.92±2.63

Note: Where FW = Fruit weight, FL = Fruit length, PT = Peel thickness, SDN = Number of seeds. Averages that do not share the same letter are significantly different at the 0.05 level. Data are averaged ± SD.

Serial Multiple Mediation Analysis using the Karlson-Holm-Breen (KHB) Method and Hayes Process Macro, Model 6:

Testing for the significance of paths a, a', b, b', c and D: Table 3 presents the results of the significance tests for **paths a, a', b, b', c,** and **D**. Path a in the model was used to evaluate the effect of peel thickness on TSS, and after adjusting for potential confounders, a positive and significant relationship was observed (β = 0.2878, 95% CI, 0.1031-0.4725). **Path b** in the model was employed to assess the impact of peel thickness on acidity, and after adjusting for confounding factors, a significant association was identified (β = 0.3134, 95% CI, 0.0270-0.5999). **Path a'** in the model was used to estimate the effect of TSS levels on vitamin C content, and after adjusting for the confounding factors, a substantial relationship was revealed (β = 37, 1728, 95% CI, 8.1949-66.1506). The **path b'** in the model was considered to evaluate the association between acidity content and vitamin C content, and again a significant association was found after adjusting for the confounding factors (β = 25.9595, 95% CI, 8.2023-43.7168). Next, path c was used to assess the effect of peel thickness on vitamin C content when mediated by both TSS and acidity levels, and yet again, a significant association was observed (β = 24.6142, 95% CI, 12.1558-37.9316). The overall relationships between peel thickness and vitamin C content were assessed based on the path D in the model. After adjusting for potential confounders, a significant and positive association was revealed (β = 31.7423, 95% CI, 54.4478-0.4171). Given that all the paths in the model were significant, a mediation analysis could be subsequently performed.

***Mediation Analysis*:** The results presented in Table 3 convey the association between peel thickness and vitamin C content when mediated by TSS and TA levels. For PT, the mediation analysis shows a significant total effect on vitamin C content of 31.7423 (95% CI, 9.0369 -54.4478; $p < 0.001$), with this comprising a direct effect of 7.1281 (95% CI, -14.1482-28.4043; $p > 0.05$) and an indirect effect of 24.6142 (95% CI, 12.1558 -37.9316). The proportion of mediation involving both TSS and acidity levels combined was found to be 77.5438%.

Table 4 shows the separate mediating effects of TSS and acidity, as well as the serial mediation effect on the relationship between peel thickness and vitamin C content. The path for peel thickness → TSS → vitamin C content reflects the mediating effect of TSS on the association between peel thickness and vitamin C content ($p<0.05$). In

Table 3. Mediation analysis results.

Testing the significance of paths, a, a′, b, b′, c, and D.				
Predictor	**Path a**		**Path a'**	**Path b**
Peel thickness	0.29(0.10; 0.47) [A]		37.17(8.19 ;66.15) [B]	0.31(0.03; 0.6) [C]
Predictor	**Path b'**		**Path c**	**Path D**
Peel thickness	25.96(8.20 ;43.72) [D]		24.61(12.16; 37.93) [E]	31.74(54.45;0.42) [F]
Médiation analysis				
Predictor	**Total Effect** (95% CI)	**Direct Effect** (95% CI)	**Indirect Effect Boot** (95% CI) ɸ	**Mediated** (%)
Peel thickness	31.74 (9.04; 54.45)***	7.13 (-14.15; 28.40)[ns]	24.61 (12.16; 37.93)	77.54

Table 4. The separate mediating effect of TSS, high TA levels, and the combination thereof on the relationship between PT and VC levels.

Predictor	PT → TSS →VC Boot (95% CI) ɸ	PT → TA → VC Boot (95% CI) ɸ	PT→ TSS → TA →VC Boot (95% CI) ɸ
Peel thickness	10.70 (2.00; 23.37)	8.14 (0.29; 16.99)	5.78 (1.17; 11.68)
Mediated Proportion %	33.704	25.633	18.207

Note: PT → TSS → VC: the mediating effect of TSS levels on the relationship between peel thickness and VC levels; PT → TA → VC: the mediating effect of TA on the association between peel thickness and VC levels; PT →TSS →TA →VC: the chain mediating effect of the combination of TSS levels and TA on the relationship between peel thickness and VC levels. The results were adjusted for fruit weight, fruit length, number of seeds, and juice yield; ɸ the significance of indirect effects was determined by the bootstrapping 95% confidence intervals. The p-values were computed accordingly. Abbreviation: CI = confidence interval, Boot: Bootstrapping. If the CI contains zero, the indirect effect is not significant.

addition, the path peel thickness → acidity content → vitamin C content indicates that acidity content mediates the relationship between peel thickness and vitamin C content ($p<0.05$). Finally, the path peel thickness → TSS → acidity content → vitamin C content indicates the effect of the serial mediation of both TSS and acidity contents on the relationship between peel thickness and vitamin C content ($p<0.05$).

DISCUSSION

***Analyzing the Variability*:** Analyzing the physicochemical characteristics of citrus fruits is crucially important for understanding the genetic diversity and variations that can influence fruit quality. In this regard, our analysis focused on several key parameters, such as weight, length, peel thickness, seed count, juice content (%), VC, TSS, and TA. According to our analysis of the variance, the FW of the *Remontant* (199.32 ± 24.34g), *Villafranca* (197.23 ± 9.45g), and *Vernia* (193.07 ± 7.54g) varieties were significantly higher than those of other varieties. These results are comparable to the study of Gulam Nabi et al. (2016) for *Corona* (159.13 g) but lower for *Mesero* (262.223 g) (Nabi *et al*., 2016), and higher for *Eureka1* (75.2 g), *Pink Variegated* (61.22 g), and *Eureka2* (71.77 g) (Sayed *et al*., 2006).

Regarding the length of the fruits, our study revealed average FL ranging from 67.11±2.84 mm for *Panaché* (the lowest) to 87.23±1.25 mm for *Vernia* (the highest). These results differ from those in another study that reported 6.28 mm for *Eureka1*, 6.25 mm for *Pink Variegated*, and 6.28 mm for *Eureka2* (Sayed *et al*., 2006). Similarly, the thickness of the peel exhibited significant variation, with the minimum PT being 3.55±0.23 mm for *Lunari* and the maximum PT being 8.23±0.15 mm for *Kerkachi*. These results differ from those in another study (Sayed *et al*., 2006) that reported thicknesses of 0.25 mm for *Eureka1*, 0.30 mm for *Pink Variegated*, and 0.27 mm for *Eureka2*.

The analysis of juice content also revealed significant variability, with there being high values for *Arbi* (40.98±0.38%), *Kennedy* (42.95±0.95%), *Lunari* (41.26±0.26%), and *Sicily* (40.22±0.22%). These results resemble those found in another study for *Corona Foothill* and *Eureka* (40.33%) (Nabi *et al*., 2016), but they are higher than values reported for *Eureka1* (22.00%), *Pink Variegated* (21.00%), and *Eureka2* (21.71%) (Sayed *et al*., 2006), as well as *Eureka* (23.13%), *Lisbon* (33.33%), and *Mesero* (36.17%) (Nabi *et al*., 2016). The other varieties in this study showed results that are very similar to those of this present work. Furthermore, our analysis of TA and TSS content resulted in total mean values of 4.95±1.18% and 7.26±0.65%, respectively, which are comparable to the 4.16% and 8.90%, respectively, reported in another study (El-Khayat, 2019).

In terms of vitamin C content, our study revealed elevated levels for *Kerkachi* (743.54±5.7 mg/L), *PSP* (736.96±5.7 mg/L), *Ba Ahmed* (733.67±5.7 mg/L), *Vernia* (694.19±5.7 mg/L), *Genes* (648.13±5.7 mg/L), *Lisbon* (687.61±5.7 mg/L), *Lunari* (608.65±5.7 mg/L), *Maghzalani* (641.55±9.87 mg/L), and *Malti* (641.55±0 mg/L), but we found lower levels for *Dellys* (493.5±0), *Valencia* (503.37±0), and *Villafranca* (427.7±5.7). These results significantly differ from those reported by another study (García Lidón *et al*., 2003), with

these ranging between 324 and 364 mg/L for *Eureka*, between 390 and 420 mg/L for *Fino*, between 383 and 409 mg/L for *Lisbon*, and between 398 and 404 mg/L for *Verna*. However, similar findings were observed for *Eureka Lemon* (El-Khayat, 2019) and *Lemon* (233.44±2.52) (Xu *et al.*, 2008).

In helping to comprehend the genetic diversity among the examined genotypes in terms of their various physicochemical parameters, these findings provide valuable insights for the selection of varieties as part of breeding programs and agricultural practices.

Multiple Mediation Analysis: This study analyzed a proposed model and found a significant positive correlation between citrus peel thickness and vitamin C content, thus confirming the prior findings of Prasad and Rao (1989) and Zandkarimi *et al.* (2011). Nevertheless, other work, most notably that of Kamatyanatti *et al.* (2016), has reported contradictory results. Likewise, Wang and Xie (2014) found that the PT of orange did not significantly affect the TSS, although a linear relationship was established between the TSS of the pulp and that of the peel. Nevertheless, the PT of citrus plays a complex role in determining VC content. For example, antioxidants in the peel can prevent VC from oxidizing, while some citrus varieties also synthesize VC in their peel, which could help increase its concentration, although it is important to note that most VC is usually found in the pulp of a fruit. What is more, VC content is influenced by various factors, such as the citrus type, variety, growing conditions, and post-harvest practices (Wang and Xie, 2014). Our results showed that fruit PT directly and positively correlates with AT content, and this is consistent with the findings of (Prasad and Rao, 1989). Nevertheless, contradictory results have also been reported, such as by(Ketsa, 1988; Zandkarimi *et al.*, 2011), who observed a negative variation in AT content as a function of PT for lime and lemon fruits. Regarding the VC content, their analysis of the direct effect of fruit PT did not reveal a significant relationship, yet our bivariate correlation analysis found there was a significant one. This is consistent with similar findings by (Zandkarimi *et al.*, 2011; Shrestha *et al.*, 2012).

During this study, a positive correlation was established between TSS content and variations in AT, and this agrees with the work of Prasad and Rao (1989), who also identified that TSS has a positive direct effect on AT content for different varieties of AT lime fruits. This finding suggests that there is a complex relationship between the level of TSS and TA, one that is potentially influenced by the regulatory mechanisms for sugar metabolism. Indeed, sugar metabolism is tightly controlled by a complex network of genes, with some promoting the synthesis of sugars and others facilitating their degradation (Katz *et al.*, 2011). However, our results differ from those reported by (Zandkarimi *et al.*, 2011; Kamlesh *et al.*, 2014), and other previous studies (Prasad and Rao, 1989; Shrestha *et al.*, 2012; Kamatyanatti *et al.*, 2016). This discrepancy, however, could be attributed to differences in the fruit varieties being studied, growing conditions, and other environmental factors that can influence sugar metabolism and AT. It is therefore imperative to conduct similar studies on a larger scale and deepen the analysis by integrating molecular regulatory mechanisms to validate and strengthen our findings. It should also be emphasized that the results we use for comparison were derived from simple bivariate correlations without taking into account potential mediators or control factors. A more in-depth approach that integrates mediation and control analyses is needed to provide a more complete understanding of the complex relationships between fruit PT, TSS, TA, and VC content. Such an approach would allow us to better understand the underlying mechanisms and establish whether there are robust links between these elements. Thus, additional research efforts are required to further enhance our knowledge of these interactions and provide a solid foundation for making future decisions and developing agricultural and nutritional interventions.

With this in mind, our study firstly aimed to examine the associations between the PT of fruits from lemon trees and the TSS, AT, and VC present in those fruits. We also sought to understand how the PT influences the level of VC content through two distinct mechanisms, so we approached this matter through two distinct paths. First, we explored the mediating role played by TSS content, so we could better understand how this intermediate variable moderates the relationship between PT and VC content. Second, we also analyzed how AT acts as an indirect mediator within this complex association, and in this way, our study attempted to develop a serial mediation model. This model aims to shed light on the complex relationship between the thickness of citrus peel and variations in VC content by taking into account the mediating effects of TSS and AT. This in-depth analytical approach therefore allowed us to better understand the underlying mechanisms that govern these complex interactions, and our findings will play a crucial role in informing decisions to select the best citrus varieties for very high VC content. By identifying key factors like PT and mediating mechanisms linked to VC levels, such as TSS and AT, we will be able to make better decisions when trying to obtain fruits that are rich in VC. Indeed, it was found that high levels of TSS tend to increase the VC content, while varieties with lower levels of TSS tend to have lower levels of VC. Similar observations have been made for citrus fruits with a high TSS content, such as the study by Alós *et al.* (2021), which examined ascorbic acid metabolism in Valencia Late oranges (Citrus sinensis) at different developmental stages. This study revealed that ascorbic acid levels in the flavedo (the outer layer of the peel) positively correlate with the L-galactose gene GGP and negatively with the ascorbic acid recycling gene DHAR1. Likewise, studies commonly observe a relationship between the concentration of vitamin C and sugar content in tomatoes (Causse *et al.*, 2003). These

findings suggest that a complex relationship exists among peel thickness, TSS, AT, and VC content in citrus fruits, highlighting the interlinked influence of several factors in the nutritional composition of fruits. The results of our mediation analysis suggest that the relationship between fruit PT and variations in VC content is fully mediated by TSS content, independently of TA levels. Indeed, our mediation model reveals a mediation percentage of 33.70% for TSS when the thickness of the citrus peel increases (Path a). This means that varieties with a thicker peel—such as Kerkachi (743.54±5.70 mg/L), Portugal (690.9±0.00 mg/L), Vernia (694.19±5.70 mg/L), Maghzalani (641.55±9.87 mg/L), and Ba Ahmed (733.67±5.70 mg/L)—tend to have higher levels of VC. On the other hand, varieties such as *Arbi*, *Kennedy*, *Corregia*, and *Lunari*, which are characterized by a thinner peel, tend to have lower levels of VC. These observations are consistent with those reported by some previous studies (Sayed *et al.*, 2006; Rekha *et al.*, 2012; El-Khayat, 2019). What is more, research by Zandkarimi *et al.* (2011) and Kamatyanatti *et al.* (2016) on lemons and limes within the citrus family revealed a positive association between TSS content, PT, and VC content, which is in line with our findings. It is important to note, however, that (Kamatyanatti *et al.*, 2016) reported a negative association specifically for acid limes, thus highlighting the complexity of the relationships at play and the need for a methodological analysis plan when exploring the causal processes among the various factors. From this perspective, it is appropriate to question whether the mediating effect of TSS could prove even more marked when selecting variants with higher TSS levels. Some research has supported this hypothesis, most notably that of Zandkarimi *et al.* (2011), who observed higher levels of VC in the M6 (370.5 mg/L) and M2 (360.8 mg/L) varieties when TSS levels reached 8.3% and 8%, respectively. In short, selecting genotypes based on the thickness of the fruit peel could have a favorable and notable impact on vitamin C enrichment within lemon fruits, so it could significantly help improve the nutritional quality of produced lemons and promote a diet rich in elements that will benefit human health.

Our study also revealed the mediating role played by high acidity in the relationship between PT and VC content, independently of TSS and other factors. Previous work, such as the study of Kamatyanatti *et al.* (2016), has demonstrated a positive correlation between AT and ascorbic acid, while other studies have reported an inverse relationship between AT and VC content, including the research of Zandkarimi *et al.* (2011) and Usman *et al.* (2020). Furthermore, our mediation analysis reveals that the indirect effect through AT constitutes 25.63% of the whole path b as PT becomes more pronounced. This finding suggests that fruits with thicker peels could have a 25.63% greater VC content when they also have higher AT levels. This effect is particularly evident in the VC contents of more AT varieties like *Kerkachi* (6.10±0.10%; 743.54±5.70 mg/L; 8.23±0.15%°Brix), *Vernia* (6.80±0.10%; 694.19±5.70 mg/L; 7.18±0.45%°Brix), *Ba Ahmed* (6.15±0.15%; 733.67±5.7 mg/L: 6.74±0.33%°Brix), and *Portugal* (6.45±0.30%; 690.9±0.00 mg/L; 7.2±0.81%°Brix).

To the best of our knowledge, although many studies have examined the associations between AT and VC content in citrus fruits, few have approached this relationship from a mediation perspective. Nevertheless, bivariate and multivariate methods have revealed significantly positive correlations between these two variables, such as in the studies of Prasad and Rao (1989) for the case of lemons and Kamatyanatti *et al.* (2016) for grapefruit varieties. Considering these observations, it is evident that selecting fruits with thicker peels can play a significant role in improving AT and VC levels, thus reinforcing the idea that citrus PT has a complex influence on the biochemical properties of fruits during maturity (Chen *et al.*, 2012), thus having direct and indirect effects on nutritional composition. Considering these factors in the breeding process could result in fruits that are more AT and richer in VC, thus contributing to agricultural products with a better nutritional quality. Additionally, our model also revealed a significant association between AT and TSS (Path c), which agrees with a study conducted by Kamatyanatti *et al.* (2016) for a group of acid limes. These findings highlight the complex nature of the interactions that exist between PT, TSS, acidity, and VC content in citrus fruits. Indeed, these factors clearly act synergistically and multidirectionally to influence the nutritional composition of fruits. Taking all of these elements together, it is clear that selecting varieties with specific profiles in terms of PT, TSS, and acidity can play a crucial role in improving the nutritional quality of citrus fruits.

Furthermore, our findings highlighted the serial mediating effect of high TSS and AT levels in the causal process that links PT to VC content. This cascade of mediations suggests that TSS and AT levels act in succession to influence the relationship between PT and VC content. Indeed, our mediation analysis revealed that a very significant 78% of the variation in VC content can be explained when considering both TSS and AT as mediators together. In addition, the work of (Chen *et al.*, 2012) highlighted the role of citric acid metabolism in carbohydrate synthesis and AT, thus reinforcing the association between high TSS levels and increased AT (Usman *et al.*, 2012). Prasad and Rao (1989) also established a link between thicker peel and higher TSS and AT levels. Taken together, these findings provide a deeper understanding of the complex interactions that take place between PT, TSS levels, AT, and VC content, and this conceptual advance could guide the development of nutritionally optimized citrus varieties. The combined influence of successive mediators reflects a complex underlying dynamic that regulates the nutritional composition of citrus fruits, thus opening the way for more in-depth approaches for selecting nutritionally optimal citrus varieties.

***Implications for Practice and Research*:** The findings of this study have crucial implications for both practical applications and future research endeavors. Firstly, identifying the significant variability among lemon accessions in terms of their physicochemical characteristics has provided valuable insights for citrus breeding programs and agricultural practices. Indeed, cultivar selection based on specific attributes—such as peel thickness, total soluble solids, acidity, and vitamin C content—could contribute to the development of citrus varieties with enhanced nutritional quality.

***Practical Implications*:** Farmers and citrus breeders can utilize the observed variations to tailor their agricultural practices and select cultivars based on specific nutritional goals. This research deepens our understanding of the relationship between peel thickness and vitamin C content, facilitating targeted interventions in citrus fruit production. Consequently, there is the prospect of cultivating fruits with improved nutritional value. The study highlights the necessity of taking into account multiple factors, including TSS and TA, when choosing citrus varieties for commercial cultivation.

***Research Implications*:** Future research should delve deeper into the molecular and genetic mechanisms that underlie the observed relationships. Molecular studies could provide a more comprehensive understanding of the factors influencing citrus fruit composition. Further research could also investigate the impact of environmental conditions on the variability of physicochemical characteristics in order to further enhance cultivation practices. Longitudinal studies could explore variations in nutritional content throughout different developmental stages, thus offering insights into optimal times for harvesting.

Strengths and Limitations

***Strengths*:** This study conducted a thorough examination of various physicochemical characteristics for different lemon accessions, thus providing a comprehensive understanding of their variability. The application of mediation analysis to explore the relationships between PT, TSS, AT, and VC content adds a novel dimension to the existing literature. The findings have direct relevance for citrus breeding programs by offering valuable information to help select genotypes with desirable nutritional profiles. The introduction of a serial mediation model contributes to achieving a more nuanced understanding of the interplay between TSS, AT, and VC content.

***Limitations*:** This study has some limitations related to the sample size, and future research could benefit from studying larger sample populations to enhance the generalizability of the findings. While the study recognizes the impact of growing conditions on the physicochemical characteristics, the specific environmental factors that influence these characteristics could be explored more comprehensively. The findings here are specific to lemon Accessions, and caution should be exercised when generalizing these results to other citrus species.

***Conclusion*:** Our study has revealed new perspectives for enriching the nutritional quality of lemons accessions by selecting varieties based on pt and TSS and AT levels in order to increase levels of VC and other beneficial compounds. These findings therefore open up exciting new avenues for improving the nutritional content of citrus fruits. In terms of the varieties considered in this study, we found that *Kerkachi*, *Portugal*, *Vernia*, *Maghzalani* and *Ba Ahmed* have higher levels of VC. Nevertheless, in-depth research that includes molecular and environmental factors is needed to better understand the complex relationships. Our findings make a start by suggesting that TSS and AT levels act as key mediators in the relationship between PT and VC content, but future studies could also help guide the selection of citrus varieties to achieve a targeted nutritional composition.

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